Sensory and behavioural responses of *Triatoma infestans* to host and conspecific odours

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(Réponses sensorielles et comportementales de *Triatoma infestans* aux odeurs de ses hôtes et de ses congénères)

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OLFACTORY AND BEHAVIOURAL RESPONSES OF THE BLOOD-SUCKING BUG *TRIATOMA INFESTANS* TO ODOURS OF VERTEBRATE HOSTS

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Summary

Olfactory receptors in basiconic and grooved-peg sensilla on the antenna of fifth-instar *Triatoma infestans* nymphs respond to host odours. Gas chromatography analyses of host odour extracts coupled to electrophysiological recordings from basiconic sensillum receptors indicate that nonanal is a constituent of sheep wool and chicken feather odour that stimulates one of the receptors in this type of sensillum. Similar analyses revealed isobutyric acid in rabbit odour to be a chemostimulant for one of the receptors in grooved-peg sensilla. The response of the aldehyde receptor was higher to heptanal, octanal and nonanal than to other aliphatic aldehydes, and the response of the acid receptor was higher to isobutyric acid than to other short-chain branched and unbranched acids.

The behavioural responses of fifth-instar *T. infestans* nymphs to nonanal and isobutyric acid in an air-stream on a servosphere indicate that, whereas nonanal causes activation of the bugs, isobutyric acid induces an increase in upwind displacement, i.e. odour-conditioned anemotaxis. Binary mixtures of these compounds did not improve the attraction obtained with isobutyric acid alone. A comparison of the behavioural and electrophysiological responses of the bugs to different amounts of isobutyric acid in air suggests that attraction is obtained at concentrations that causes low-to-moderate increases in the firing rate of the acid-excited receptor in the grooved-peg sensilla, whereas at a dose that evokes relatively high firing rates (>40 Hz) no attraction is obtained.

Key words: *Triatoma infestans*, *Rhodnius prolixus*, Triatominae, olfactory receptor, odour, behaviour, insect vector, olfaction.

Introduction

Haematophagous reduviid bugs, also known as triatomine bugs, have served as models for studies on insect physiology for well over half a century (Wigglesworth and Gillett, 1934). *Triatoma infestans* (Klug) the most studied species, is one of the main vectors of American trypanosomiasis or Chagas' disease in South America. Adults and larvae of this insect occupy predominantly domestic habitats, although they are also associated with peridomestic chicken coops, guinea-pig runs and goat and sheep corrals (Schofield, 1994; Gürtler et al., 1996). They feed at night when their hosts are asleep and take refuge in crevices of dwellings during daylight.

Triatomines use different sensory cues to locate hosts, one of which is host odour. Host volatiles attracted triatomine bugs on a servosphere when delivered in complete darkness in an air-stream under conditions of constant temperature and humidity, suggesting that host finding can be achieved by the use of olfactory cues alone (Taneja and Guerin, 1995). CO_2 has been shown to play an important role in host location by triatomines (Taneja and Guerin, 1995), as is the case for other haematophagous arthropods. However, other components of vertebrate odour were implicated in host-finding by triatomines since the attraction to host odour was stronger than to CO_2 alone (Taneja and Guerin, 1995). The odours of stale host urine and one of its components, ammonia, were found to attract *T. infestans* (Taneja and Guerin, 1997). Furthermore, a complex blend of volatiles, including CO_2, produced by the aerobic growth of baker's yeast, successfully lured triatomines to traps in the laboratory (Guerenstein et al., 1995) and under natural conditions (Lorenzo et al., 1998).

To date, the function of two morphologically distinguishable types of olfactory sensilla (Fig. 1) have been studied on the antenna of triatomines. Electron microscopy indicates that the single-walled wall-pore basiconic sensilla are non-articulated and 30 μm long, with a high density of wall pores linked to pore tubules (Bernard, 1974). These sensilla are multi-innervated (21–41 receptor cells) with extensive dendritic branching in the sensillar lumen (M. Vlimant, P. A. Diehl, R. A. Steinbrecht, P. G. Guerenstein and P. M. Guerin, unpublished data). Receptors within this sensillum type on adults were reported to respond to human breath, pyridine and furan (Mayer, 1968). The other morphological type, i.e. the double-walled wall-pore grooved-peg (GP) sensillum, is a non-articulated longitudinally grooved 8–18 μm long peg, with wall pores that communicate *via* spoke channels crossing the double cuticular wall to the central lumen of the sensillum (Bernard, 1974). In these sensilla, there are 4–5 receptor cells without

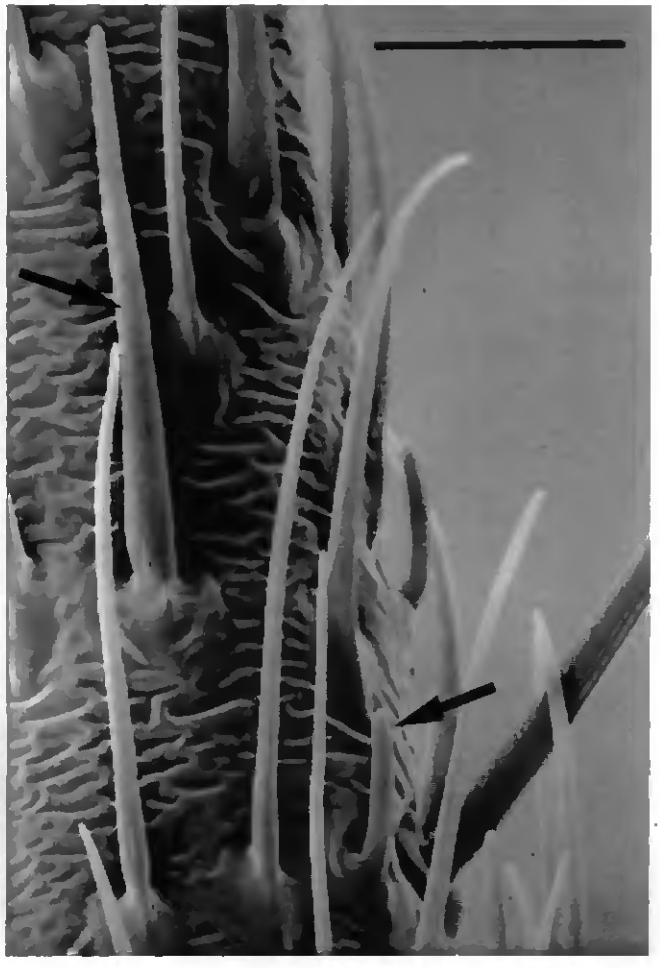

Fig. 1. Scanning electron micrograph of a basiconic sensillum (arrow upper left) and a grooved-peg sensillum (arrow lower right) on the terminal antennal segment of a fifth-instar *Triatoma infestans* nymph. Scale bar, 20 µm.

dendritic branching (M. Vlimant, P. A. Diehl, R. A. Steinbrecht, P. G. Guerenstein and P. M. Guerin, unpublished data). Single-sensillum electrophysiological recordings indicate that two functionally different GP sensillum types exist on the antenna of nymphs: type 1 (GP 1) with an NH_3-excited receptor neurone, and type 2 (GP 2) housing an NH_3-excited cell in addition to a neurone responding to short-chain fatty acids (Taneja and Guerin, 1997).

Here, we recount how gas chromatography analyses of host odour extracts were coupled to electrophysiological recordings from olfactory receptors in the basiconic and GP 2 sensilla to isolate and identify chemostimulants for the triatomines in host odour extracts, and we also describe the behavioural responses of the bugs running on a servosphere in response to these and other vertebrate-associated volatiles.

Materials and methods

Insects

A colony of *Triatoma infestans* (Klug) was maintained at 28.5±0.5 °C, 50±5 % relative humidity (RH) and on a 12 h:12 h L:D cycle in a climate chamber as described previously (Taneja and Guerin, 1997). Fifth-instar *T. infestans* nymphs (unless stated otherwise) were used in the behavioural and electrophysiological experiments described here. Upon moulting to the fifth instar, each nymph was placed in a climatic chamber at 19±0.5 °C, 80±5 % RH and on a 12 h:12 h L:D cycle until it was used in experiments. Behavioural experiments were performed with nymphs starved for 6–11 weeks post-ecdysis, and 3- to 8-week-old nymphs were used for electrophysiological recordings.

Electrophysiology

The method of recording responses from triatomine olfactory sensilla was as described by Taneja and Guerin (Taneja and Guerin, 1997). The antenna was bathed in a humidified air-stream (90–100 % RH, 23±2 °C) delivered at 1 m s^{-1} *via* a glass water-jacketed tube (6 mm internal diameter) whose outlet was approximately 1 cm from the preparation. The electrophysiological signal was fed into an AC/DC amplifier (UN-03, Syntech, The Netherlands) *via* a high-impedance preamplifier (Syntech), recorded on the hard disk of a PC *via* a 16-bit analog/digital IDAC card (Syntech) and monitored simultaneously with an oscilloscope (Tektronix 5103, USA). Action potentials were analysed using the spike analysis programme Autospike (Syntech).

Stimulus delivery for electrophysiological recordings

Stimulation was as described by Taneja and Guerin (Taneja and Guerin, 1997). A sample of the stimulus solution was deposited on a 0.8 cm×3 cm filter paper strip and placed in the 5 ml stimulus syringe cartridge after evaporation of the organic solvent. In the case of synthetic products, 0.001–100 µg doses of a chemical dissolved in 10 µl of solvent (see below) were used. Stimulus evaporation within the stimulus cartridge was allowed to occur for 3 min prior to use. CO_2 was delivered by diverting the gas flow from a gas cylinder (2 % v/v CO_2 in O_2) through the cartridge. The stimulus puff (1 s) was introduced through a septum-covered hole (24 cm from the preparation) in the water-jacketed tube (see above). Intervals between stimulations were at least 3 min. For dose/response curves, odours were presented at increasing doses in logarithmic steps. Regression analyses using a linear model were used to analyse response trends to individual products, and regression comparisons were made according to Hald (Hald, 1967) to compare dose/response trends to different products. This comparison tests differences in the slopes of the response functions; when the slopes are not significantly different, the intercepts with the ordinate (i.e. the relative levels of response) are compared.

Natural and synthetic stimuli for electrophysiological recordings

Basicanic sensillum

Host odour concentrates, odours of conspecifics and synthetic chemicals were tested to study the response profiles of the

olfactory receptors in basiconic sensilla. Host odours included sheep wool, chicken feathers and rabbit odour extracts (see below), human breath and human axillary odour. Disturbed *T. infestans* adults and triatomine faeces were also tested. To collect vertebrate odours, charcoal-filtered air was sucked with a water pump for 3 h at 400 ml min^{-1} through a 21 desiccator containing either 70 g of sheep wool (collected 4–12 weeks before and stored at −80 °C) or 60 g of chicken feathers (freshly collected) to a Pasteur pipette (5 mm internal diameter) containing 460 mg of preconditioned (Byrne et al., 1975) Porapak Q (60–80 mesh, Millipore Corporation, USA). The desiccator was placed in a waterbath at 37 °C. Volatiles were desorbed from the adsorbent with 500 µl of dichloromethane (Merck, analytical grade). The chicken feather extracts were concentrated to 50 µl, and samples of 5 or 10 µl were placed in the stimulus cartridge. The sheep wool extracts were not concentrated, and a 20 µl sample was placed in the stimulus cartridge. Air from the desiccator without host material was also collected as described above (blank control). Rabbit odour extracts were prepared on Porapak Q as described by Steullet and Guerin (Steullet and Guerin, 1994a). Volatiles were desorbed with 3 ml of dichloromethane, and the extracts were concentrated to 50 µl, of which 20 µl samples (5 µl when tested on GP2 sensilla, see below) were placed in the stimulus cartridge. Air from the corridor outside the rabbit room was also sampled for a similar period (blank control). Human breath and human axillary odour were collected and used as stimului as described by Steullet and Guerin (Steullet and Guerin, 1992). The stimulus blank was humidified air. Disturbed adult *T. infestans* (two males and one female) were placed in a stimulus cartridge and shaken for some seconds just before stimulation, and faeces of *T. infestans* nymphs were tested (see Taneja and Guerin, 1997). Synthetic chemicals, dissolved in either dichloromethane or water, belonging to different chemical classes were tested at 10–100 µg in the stimulus cartridge. Compounds tested included C_6–C_{12} saturated aliphatic aldehydes, *trans*-2-hexenal, *cis*-3-hexenal, *trans*-2-heptenal, *trans*-2-nonenal, 2,6-nonadienal, 2,4-decadienal, nonane, nonanol, nonanoic acid, 2-nonanone, pyridine and 4-hydrofuran.

Grooved-peg 2 sensillum

Stale rabbit urine, human foot swabs and Emmental cheese were tested in addition to odour extracts of chicken feathers and rabbits, mechanically disturbed adult *T. infestans* and triatomine faeces. Odours of human feet were collected by leaving a filter paper strip (2 cm×6 cm) for 30 min in the socks being worn by men. The following synthetic compounds, dissolved either in dichloromethane or in water, were tested: NH_4OH (0.1 % in distilled water), propanoic acid, butyric acid, isobutyric acid, 2-methylbutyric acid, pentanoic acid, isovaleric acid, L-(+)-lactic acid and 2-hydroxybutyric acid. The compounds were tested at 10 µg in the stimulus cartridge with the exception of isobutyric acid, which was tested at 1 µg. All chemicals tested on basiconic and GP2 sensilla, except ammonia, were 97 % pure; dichloromethane and distilled water were used as solvent blanks.

Gas-chromatography-coupled electrophysiological recordings (GC-EL)

The methodology was described by Steullet and Guerin (Steullet and Guerin, 1994a). Triatomine olfactory receptors responding to odours of vertebrates or of conspecifics were employed as detectors in tandem with gas chromatography to locate biologically active constituents of odour extracts. The components of an active extract were separated on a high-resolution gas chromatography capillary column in a Carlo Erba Instruments 5160 gas chromatograph with an on-column injector and a flame ionisation detector (FID, at 260 °C). The sheep wool extract was injected on-column to a 25 m fused-silica SE-54 column (Macherey-Nagel, Switzerland; internal diameter 0.25 mm, film thickness 0.35 µm); the carrier gas was H_2 flowing at 27 cm s^{-1} at 40 °C. Injections were made at 40 °C, and the column temperature was programmed to increase at 8 °C min^{-1} to 240 °C. The rabbit and chicken feather odour extracts were injected on-column to a 30 m fused-silica DBWAX column (J&W Scientific, USA; internal diameter, 0.25 mm; film thickness 0.25 µm); the carrier gas was H_2 flowing at 46 cm s^{-1} at 40 °C. Injections were made at 40 °C, and the column temperature was programmed to increase at 8 °C min^{-1} to 230 °C. The column effluent was split (glass Y-splitter), 60 % to the flame ionisation detector and 40 % to the electrophysiological preparation. The latter was swept by the humified air-stream (see above) to the electrophysiological preparation from a heated transfer line (240 °C) in the wall of the chromatograph in such a way that the column effluent was simultaneously monitored by the flame ionisation detector and triatomine olfactory receptors.

Action potentials recorded from sensilla employed as biological detectors were sorted from background noise by a level discriminator incorporated in the UN-03 amplifier (above), and the sum of the frequencies of all firing cells was continuously converted to a voltage (the time constant of the frequency-to-voltage converter was 0.1 s). This signal and the flame ionisation detector response were recorded simultaneously on a PC using a software program (GC-EAD, Syntech). The Kovat's index for a biologically active component was calculated with reference to *n*-alkanes (C_{10}–C_{24}) injected under the same gas chromatography conditions as chemicals being analysed. The action potentials were simultaneously recorded on a tape in a DAT recorder (Biologic, France) for subsequent analysis using Autospike (see above).

Gas-chromatography-coupled mass spectrometry (GC-MS)

Odour extracts identified as having biologically active constituents by GC-EL were subsequently concentrated (rabbit twice, sheep wool 20 times) and analysed by GC-MS in a Hewlett Packard 5890 series II chromatograph (column conditions as in GC-EL) with a mass-selective detector (HP 5971A). The chicken feather extract was analysed by GC-MS on a free-fatty-acid-phase capillary column (30 m FFAP, 0.25 mm internal diameter, 0.25 µm film thickness, BGB Analytik, Switzerland). Blank controls were analysed using the

same procedure as for the respective host odour extracts. Extract (2 µl) was injected on-column connected *via* 1 m of deactivated fused-silica capillary to the mass spectrometer (ionisation energy 70 eV) with helium as carrier gas at constant flow (linear velocity approximately 30 m s^{-1} at 40 °C). Active components of the extracts located by GC-EL were relocated in GC-MS using the Kovat's indices and by comparison of chromatogram profiles obtained in GC-EL and GC-MS. Identification of an electrophysiologically active peak in an extract was first based on the match of its mass spectrum with that of a known product stored in a computer-based library using the HP CHEMSTATION. The Kovat's index of the unknown was then compared with that of the library-proposed synthetic analogue injected under the same conditions. The biological activity of synthetic analogues (≥97 % purity) was confirmed by electrophysiological assay on the relevant olfactory receptors. Octanal, nonanal and isobutyric acid were employed as standards in GC-MS and electrophysiology.

Servosphere

The method of recording the behavioural responses of triatomine bugs to volatile chemostimulants using the servosphere was described by Taneja and Guerin (Taneja and Guerin, 1995). Tests were made in the dark at 24±1 °C during the scotophase of the daily cycle of the bugs. Experiments started at least 10 min after the animal had been placed on the sphere to allow it to acclimate to the test environment. An experiment consisted of three 2 min periods, i.e. control, test and end-control. Two different protocols were followed: when dichloromethane was the solvent for the chemostimulants (see below), the three periods of an experiment were recorded consecutively (as in Taneja and Guerin, 1995). When paraffin oil was the solvent, the test and end-control periods were recorded consecutively, approximately 1 min after the end of the control period, such that the control and test periods started only when the bugs were walking downwind or crosswind (for a definition of upwind walking, see below). In this manner, the walking direction of the bug with respect to wind at the start of the test period was approximately the same as for the control.

Track analysis

The *x,y* coordinates of displacements by the bugs were recorded (as in Taneja and Guerin, 1995) every 0.1 s at a base resolution of 0.1 mm on a SAMII 68 K computer (KWS Inc., Ettlingen, Germany) *via* pulse generators responding to the displacements of the servosphere. These coordinates, constituting instantaneous vectors with a length and a direction with respect to wind (0° is straight upwind), were analysed (after discarding all instantaneous displacements smaller than 0.5 mm) on a PC using track-analysis software (Taneja and Guerin, 1995). The track vectors were used to calculate the walking speed of the bugs for the control, test and end-control periods of an experiment for each insect. The increase in the percentage distance walked in the upwind cone (defined arbitrarily as 60° either side of due upwind) in the test over the percentage recorded for the preceding control period of equal duration was used as a means of measuring attraction and, when significant, was termed an 'upwind response'. A 'target vector' was also calculated by multiplying the cosine of the mean direction ϕ (mean angle of Batschelet, 1981) of all vectors with respect to wind by the path straightness r (directly proportional to the variance of ϕ; Batschelet, 1981). Whereas r varies between 0 and 1 (1 indicating a straight path), the target vector varies between −1 and 1 (1 indicating walking straight upwind). The latter parameter incorporates an estimate of the mean direction of the path and the consistency with which that particular direction is maintained irrespective of the arbitrarily chosen upwind cone. Differences between walks in test and control periods were analysed pair-wise using the two-tailed Wilcoxon signed-rank test.

Stimuli and stimulus delivery for behavioural recordings

Dilutions of the compounds tested were made in dichloromethane, paraffin oil (Merck, spectroscopy grade) or distilled water. Test chemical solutions (100 µl) were applied to filter paper discs (90 mm diameter) placed upright at the bottom of a 500 ml gas-wash bottle. Controls consisted of bottles loaded with the same amount of solvent on filter paper. Dichloromethane was left to evaporate before placing the filter paper in the bottle. Gas-wash bottles were then left to equilibrate for at least 10 min before presenting the evaporated vapours to the bugs on the servosphere. During evaporation of dichloromethane, an unknown amount of test chemical was also lost, and this effect was more critical at low stimulus doses. The use of paraffin oil as a solvent has the advantage of retarding the release of the test chemical, thus minimising loss. Filter papers for both the control and test gas-wash bottles were replaced after each test. The stimulus delivery system to the apex of the servosphere was described by Taneja and Guerin (Taneja and Guerin, 1995). The behavioural responses to nonanal, isobutyric acid, CO_2, human breath, mixtures of nonanal plus isobutyric acid, CO_2 plus isobutyric acid and NH_3 plus isobutyric acid were tested. All chemicals were at least 97 % pure. Dilutions of NH_4OH were made in water. CO_2 was tested as for the electrophysiological experiments (see above) by diverting the gas flow into the conditioned air-stream passing over the apex of the servosphere. Human breath from an adult male was blown into a 500 ml gas-wash bottle for 30 s, and the bottle was left to stand upright for at least 20 min before the start of a test. Introduction of the vapours from the gas-wash bottle into the main air-stream diluted the breath 25-fold, and this provided a stable (1500 p.p.m.) level of CO_2 at the apex of the sphere for 1 min as measured by an infrared gas analyser (BINOS 1, Leybold-Heraeus, Germany). Distilled water (1 ml) was introduced into the control flask for breath to preclude a shift in humidity during the test period. To detect any spontaneous tendency of the animals to change their behaviour during the tests carried out with dichloromethane as solvent, an experiment was performed in which dichloromethane alone was applied to the filter papers and evaporated (as above) before placement in the gas-wash bottles.

Estimation of release rates of isobutyric acid and nonanal onto the servosphere

Synthetic air (80 % N_2 and 20 % O_2) at 150 ml min^{-1} was passed for 2 min through a 500 ml gas-wash bottle containing one filter paper disc (diameter 90 mm) loaded with a given amount of either isobutyric acid or nonanal. The air leaving the bottle passed through a charcoal trap (1.5 mg; Grob and Zürcher, 1976) in a 4 mm diameter glass tube where the volatiles were collected 10 min after introducing the filter papers to the bottle. To elute the adsorbed odours, approximately 15 µl of dichloromethane was placed on the charcoal bed and, by alternately generating positive and negative pressure *via* a glass syringe attached to the glass tube, the solvent was flushed successively through the adsorbent. Approximately 8 µl of solvent was recovered; of this, 2 µl was injected onto a fused silica DB-WAX gas chromatography capillary column (temperature programmed as described above). The recovery of volatiles was quantified by comparing peak areas with those of known quantities of standards injected under the same conditions using an integrator (Spectra-Physics, USA). Corrections were made to compensate for amounts of compounds remaining on the charcoal trap after the first elution.

Results

Recordings from the base or tip of basiconic sensilla on the antenna of 3- to 8-week-old fifth-instar *T. infestans* nymphs revealed spontaneous activity in a number of cells (more than 6). These receptors always showed an increase in spike frequency in response to stimulation with sheep wool, rabbit (N=8 for sheep wool and N=3 sensillar recordings for rabbit odour; Fig. 2) and chicken feather (N=3) odour, as collected on Porapaq Q, and to human axillary odour (N=5). Other vertebrate odours, such as human breath (N=7), did not activate any receptors within this sensillum type. In the GP sensilla, 3–5 receptors generating action potentials of different amplitudes were spontaneously active. These receptors always showed an increase in action potential frequency in response to stimulation with rabbit (N=9; Fig. 3) and chicken feather (N=5) odour, as collected on Porapak Q, and to the odours of mechanically disturbed *T. infestans* adults (N=3), Emmental cheese (N=7; Fig. 3) and human foot odour (N=2).

Vertebrate volatiles activating basiconic sensillum receptors

Gas-chromatography-coupled electrophysiological recordings (GC-EL) from single sensilla revealed one clearly active component of sheep wool and chicken feather odour that stimulated an olfactory receptor in the basiconic sensillum (Fig. 4). Using gas-chromatography-coupled mass spectrometry (GC-MS), this active volatile was identified as nonanal at 6 ng $µl^{-1}$ and 12 ng $µl^{-1}$ in the sheep wool and chicken feather extracts, respectively (Table 1). Octanal, present at 1 ng $µl^{-1}$ in the sheep wool odour extract, also activated a receptor with spikes of the same amplitude as those evoked by nonanal in GC-EL analysis of sheep wool odour (Table 1). Although rabbit odour was not analysed by GC-EL, the spike amplitude of the cell responding to the whole odour extract was the same as that of the cell responding to nonanal (Fig. 2). GC-MS analysis of the rabbit odour extract detected nonanal at 4 ng $µl^{-1}$, a level that could account for the electrophysiological response. A response to synthetic nonanal was recorded in all

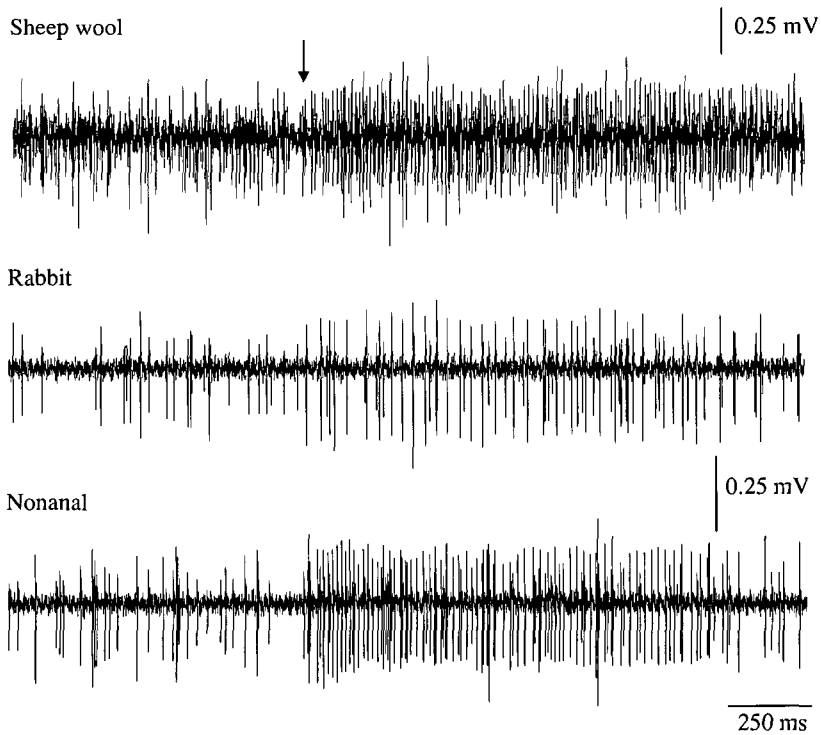

Fig. 2. Responses to sheep wool and rabbit odour and to nonanal as one of their constituents (5 µg in the stimulus cartridge) of olfactory receptors in basiconic sensilla on the antenna of a fifth-instar *Triatoma infestans* nymph. The response to sheep wool was from a different preparation from that used for the other two stimuli; such variability in the nature of the spike activity captured from this sensillum was normal. The increase in the summed frequency of all action potentials over the response to the blank control was 41, 16 and 58 Hz, respectively, for the three treatments tested. The arrow indicates the start of stimulation for 1 s.

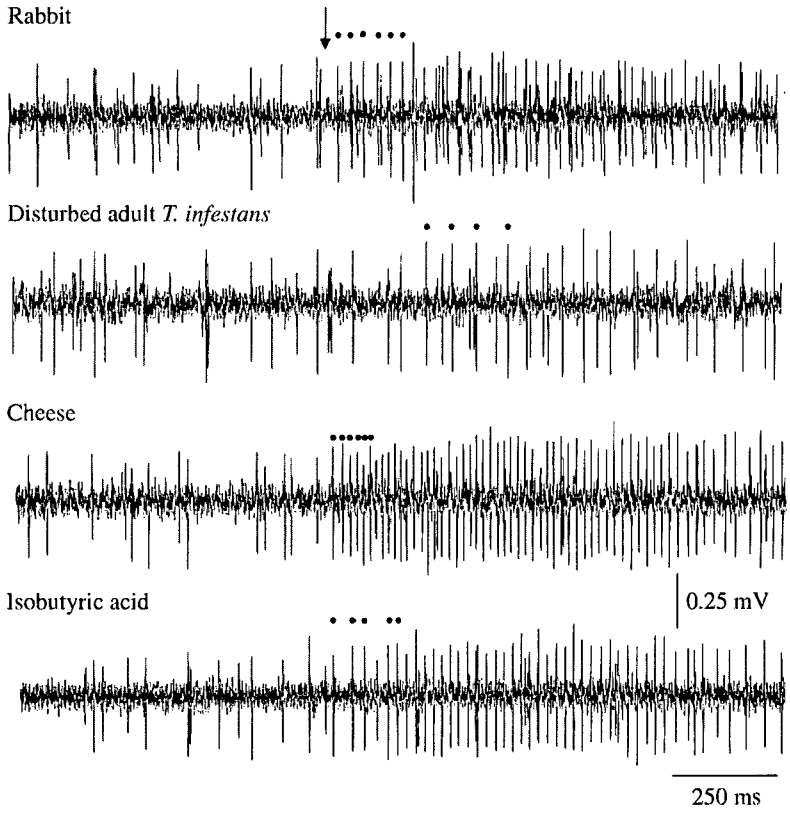

Fig. 3. Responses recorded from a GP2 olfactory sensillum on the antenna of a *Triatoma infestans* fifth-instar nymph to odour of rabbit as collected on Porapak Q, to three adult *T. infestans*, to 3 g of Emmental cheese and to isobutyric acid (100 ng) in the stimulus cartridge. The action potentials marked with a filled circle in each trace are from an acid-sensitive cell, showing an increase in frequency over the response to the blank control of 16, 7, 34 and 22 Hz, respectively, for the four treatments tested; the arrow indicates the start of the 1 s stimulation.

stable preparations of the basiconic sensilla (N>40). In some preparations, it was clear that only one cell responded to the compound, but in others at least one other cell with a smaller spike amplitude also increased its firing rate in response to stimulation with nonanal. Moreover, the relative amplitude of the action potential of the neurone most clearly responding to aldehydes was variable from one sensillum to another. Because of this and the multicellular nature of recordings from the basiconic sensilla, all action potentials were counted when quantifying responses from this sensillum type.

The response of the olfactory receptors in the basiconic sensilla to saturated aliphatic aldehydes showed dose-dependency within the range tested for heptanal, octanal and nonanal (P<0.001 for all regression lines, Fig. 5). The trend

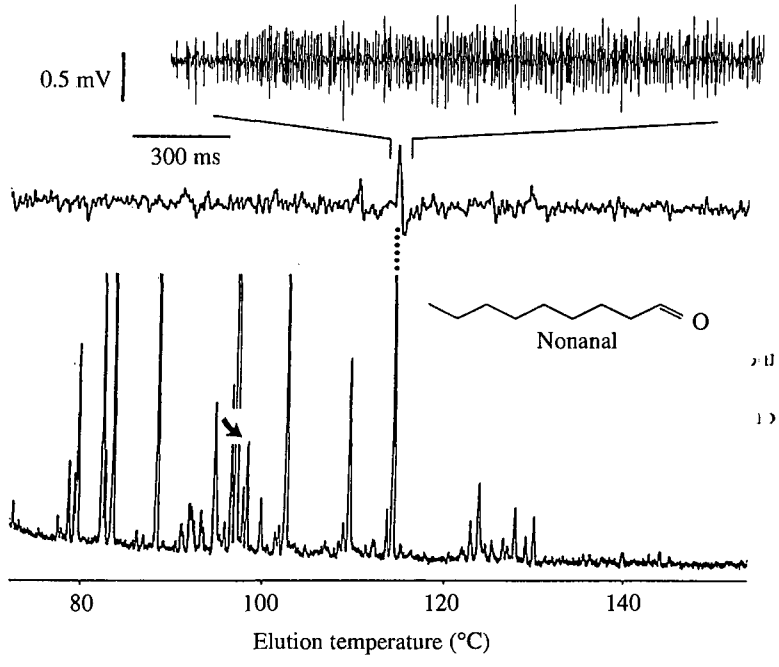

Fig. 4. Analysis of sheep wool odour collected on Porapak Q by gas chromatography coupled to an electrophysiological recording from olfactory receptors in a basiconic sensillum on the antenna of a *Triatoma infestans* fifth-instar nymph. The lower trace is the flame ionisation detector response and the middle trace is the summed frequency of all firing cells (frequency-to-voltage-converted signal). The upper trace is the actual spike train generated during elution of the biologically active constituent of the extract from the gas chromatograph at 114 °C. The active peak (approximately 12 ng) was identified as nonanal because the activated receptor responded to a similar amount of synthetic nonanal at the elution temperature of the active product in the extract. The arrow indicates octanal (approximately 2 ng), which was also found to activate the aldehyde receptor of a basiconic sensillum in a separate analysis of sheep wool odour.

Table 1. *Constituents of vertebrate odour that stimulate olfactory receptors in basiconic and grooved-peg type 2 sensilla on the antenna of fifth instar* Triatoma infestans *nymphs*

Sensillum type	Olfactory stimulant	Odour source	Identification criteria[a]	Kovat's index of active peak[b]	Kovat's index of synthetic analogue[c]	Response occurrence[d]
Basiconic	Octanal	Sheep wool	ME	1006	1004	1/4
	Nonanal	Sheep wool	ME	1118±1.5	1116	4/4
		Chicken feathers	MRE			1/1
Grooved-peg type 2	Isobutyric acid	Rabbit	ME	1572±14	1560±2	3/3

This table is based on gas-chromatography-coupled electrophysiological (GC-EL) olfactory sensillum recordings and gas-chromatography-coupled mass spectrometry (GC-MS) analyses of sheep wool, chicken feather and rabbit odours as collected on Porapak Q.

[a]Criteria on which identification of a biologically active vertebrate volatile was based: M, matching mass spectrum; R, matching retention time of the synthetic analogue; E, matching electrophysiological activity with that of the synthetic analogue.

[b]Kovat's index of the electrophysiologically active peaks.

[c]Kovat's index established by GC-MS of the synthetic analogues that matched the biological activity of the active peak in GC-EL.

[d]Number of GC-EL analyses in which a response to the compound was observed from the total number of such analyses of the particular odour extract.

Values are means ± S.D. where three or more analyses were made.

lines for the C_7–C_9 aldehydes did not differ, as indicated by comparison of the regressions. No such responses were recorded for hexanal, decanal, undecanal and dodecanal. A nonanal-excited cell was also found in basiconic sensilla of *T. infestans* first-instar nymphs and adults and in fifth-instar nymphs and adults of two other triatomine species, *Rhodnius prolixus* and *Dipetalogaster maxima* (results not shown). Compounds of different functionality were also tested on basiconic sensilla of fifth-instar *T. infestans* nymphs to examine further the specificity of the aldehyde-excited receptor(s), but no response was obtained to nonane, nonanol, 2-nonanone or nonanoic acid or to the unsaturated aliphatic aldehydes (*Z*)-3-hexenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-nonenal, 2,6-nonadienal and 2,4-decadienal.

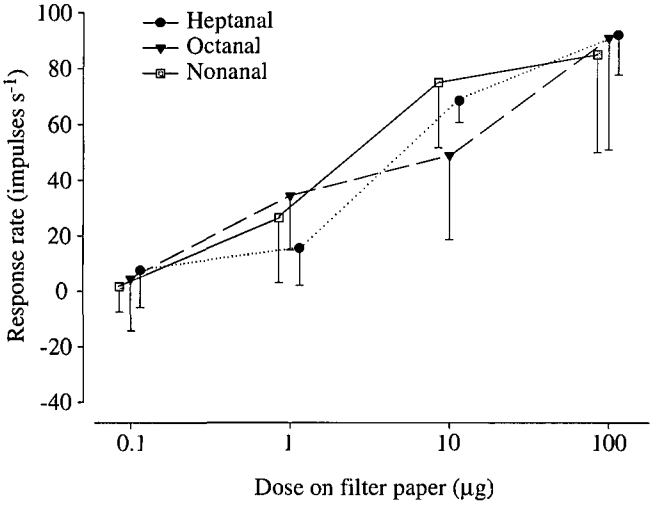

Fig. 5. Response curves of aldehyde receptors in basiconic sensilla on the antennae of *Triatoma infestans* fifth-instar nymphs to a 1 s stimulation with different doses of C_7–C_9 aliphatic aldehydes. Ordinate, summed frequency of all cells firing in the first second of the response after subtraction of the blank control value [because of the multicellular activity in this sensillum type, the spike frequency of the responding receptor(s) could not be properly quantified on their own]; abscissa, dose of aldehyde in the stimulus cartridge. The electrophysiological response increased significantly with dose within the range tested for heptanal, octanal and nonanal, and the response trends are the same (see text). Some preparations clearly responded to nonanal at 0.1 μg in the stimulus cartridge. Values shown are means and standard deviations; N=4 for each compound. Mean frequencies for a given dose have been displaced slightly for clarity.

Vertebrate volatiles activating grooved-peg sensillum receptors

The GP2 sensillum type with the NH_3-sensitive receptor accompanied by a cell responding to short-chain fatty acids was easily identified throughout this study. GC-EL showed that one component of rabbit odour stimulated the fatty-acid-sensitive olfactory receptor within this sensillum type (Fig. 6), and this volatile was identified as isobutyric acid at 10 ng μl^{-1} using GC-MS (Table 1). Although the rabbit odour extract was also shown to contain propionic acid at 6 ng μl^{-1}, this compound never activated the acid-sensitive cell during GC-EL experiments. The spike amplitude of the cell activated by odours of disturbed bugs, cheese (Fig. 3), human feet and chicken feathers (not shown) was the same as that of the cell responding to isobutyric acid.

The response of this acid-sensitive receptor in the GP2 sensillum to different short-chain fatty acids showed dose-dependency within the concentration range tested for isobutyric, butyric and isovaleric acids (P<0.005 for all regression lines, Fig. 7), with common slopes. However, the response to isobutyric acid was the highest throughout the dose range tested (P<0.001), indicating a lower threshold for this product. 2-Methylbutyric acid was also tested on two

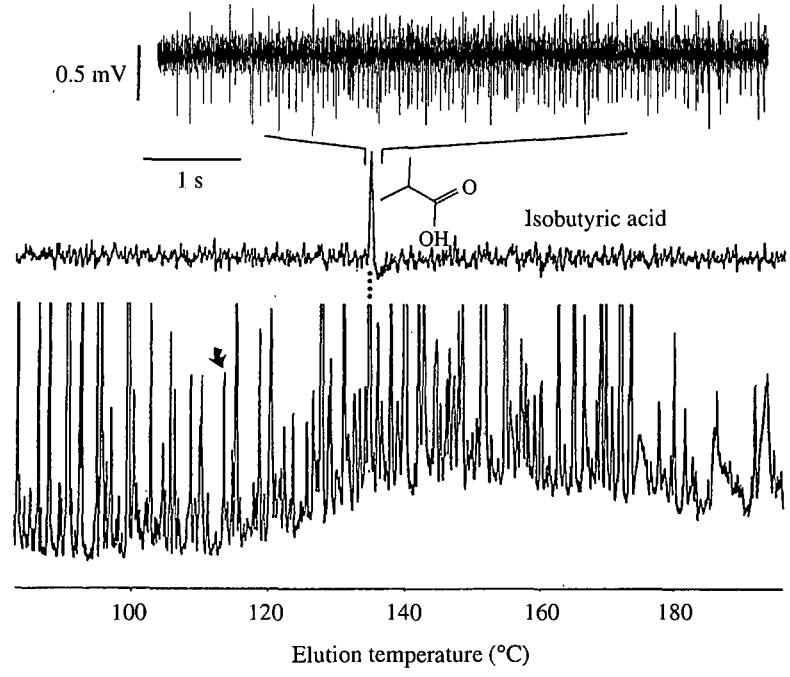

Fig. 6. Analysis of rabbit odour collected on Porapak Q by gas chromatography coupled to an electrophysiological recording from olfactory receptors in a GP2 sensillum on the antenna of a *Triatoma infestans* fifth-instar nymph. The lower trace is the flame ionisation detector response and the middle trace is the summed frequency of all firing cells (frequency-to-voltage-converted signal). The upper trace is the actual spike train generated during elution of the biologically active constituent of the extract from the gas chromatograph at 134 °C. The active peak (approximately 20 ng) was identified as isobutyric acid because the activated receptor responded to a similar amount of synthetic isobutyric acid at the elution temperature of the active product in the extract. The arrow indicates the nonanal peak (approximately 4 ng) that activates an aldehyde receptor in basiconic sensilla on triatomine antennae.

preparations at 0.1 and 1 μg in the stimulus cartridge (results not shown), and the response hardly differed from that of isovaleric acid (3-methylbutyric acid). The acid-sensitive receptor did not respond to L-(+)-lactic acid at any of the doses tested (Fig. 7) and, in addition, showed only weak responses to pentanoic, propanoic and 2-hydroxybutyric acid at 1 μg in the stimulus cartridge.

Behavioural responses to vertebrate volatiles

Two previously identified attractants for triatomines (Taneja and Guerin, 1995; Taneja and Guerin, 1997) were tested as positive controls. The response to 1000 p.p.m. CO_2 in the air over *T. infestans* fifth-instar nymphs on the servosphere was characterised by an increase in walking speed during the test with respect to the control period (median of the increase 1.5 mm s^{-1} over the control value of 26.8 mm s^{-1}; $P<0.05$, $N=11$). In addition, the nymphs walked significantly more upwind (median of the increase 20 %, $P<0.05$). Ammonia at 1 part per billion (p.p.b.) in the air streaming over adult *T. infestans* on the servosphere also caused an upwind response (median of the increase 40 %, $P<0.05$, $N=10$). At a source dose of 10 μg, nonanal caused a significant increase in the walking speed of *T. infestans* nymphs with respect to the control (median of the increase 2.3 mm s^{-1} over the control value of 22.1 mm s^{-1}; $P<0.05$, $N=10$), but this effect was not apparent at a source dose of 1 μg ($N=10$). No increase in speed was observed when nonanal was dissolved in paraffin oil at a source dose of either 50 ($N=16$) or 200 μg ($N=14$). With dichloromethane as solvent, the concentration of nonanal in the air was estimated to be 1 and 8 p.p.b. for source doses of 1 and 10 μg, respectively, and 3 p.p.b. for a source dose of 50 μg in paraffin oil (results not shown).

Isobutyric acid did not significantly affect the walking speed of *T. infestans* nymphs at any of the doses tested, but this

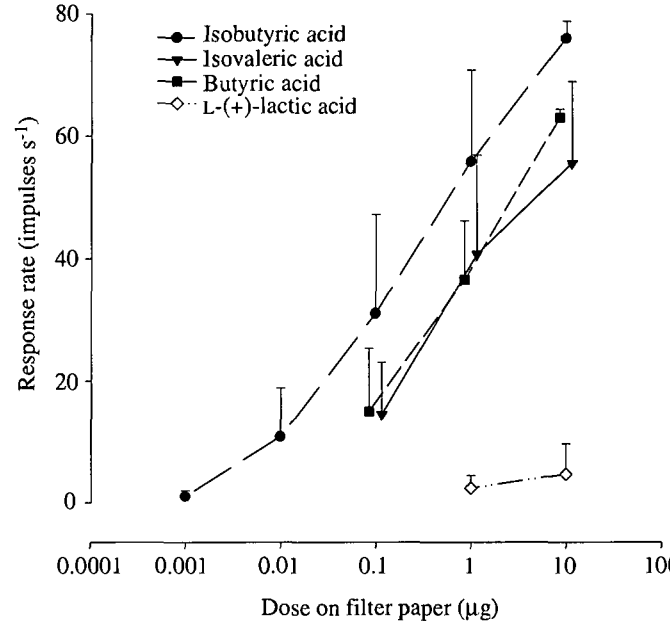

Fig. 7. Response curves of the fatty acid receptors in GP2 sensilla on the antennae of *Triatoma infestans* fifth-instar nymphs to a 1 s stimulation with different doses of short-chain fatty acids. Ordinate, firing frequency of the responding receptor cell in the first second of the response after subtraction of the blank control value; abscissa, dose of fatty acid in the stimulus cartridge. The response of the acid receptor increased significantly with dose within the range tested for isobutyric, butyric and isovaleric acids, but not for L-(+)-lactic acid. A regression analysis indicated that the response to isobutyric acid was significantly greater than to the other acids (see text). Values are plotted as means and standard deviations; $N=3$ for lactic acid, and $N=6$ for the other acids except for doses of 0.001 μg ($N=3$) and 10 μg ($N=2$). The mean frequencies for a given dose have been displaced slightly for clarity.

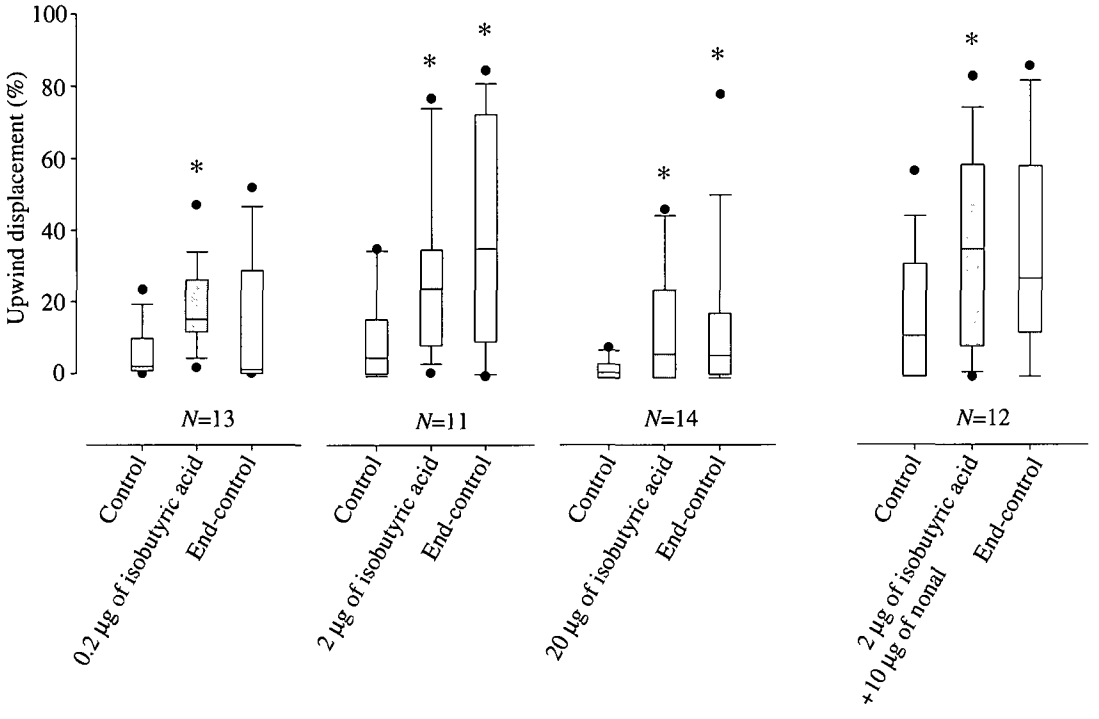

Fig. 8. Box plot representation of distance walked in a cone 60° either side of due upwind on a servosphere by *Triatoma infestans* fifth-instar nymphs as a percentage of the total distance walked in separate control, test and end-control periods of tests in which the responses to different doses of isobutyric acid and a binary mixture of isobutyric acid plus nonanal dissolved in dichloromethane were tested. An asterisk indicates that the percentage upwind displacement was significantly greater in that period than in the corresponding control period ($P<0.05$, two-tailed Wilcoxon signed-rank test for paired replicates). The lines within a box mark the median, the lower and upper boundaries of a box indicate the twenty-fifth and seventy-fifth percentiles, the bars below and above a box indicate the tenth and ninetieth percentiles and the filled circles represent the fifth and ninety-fifth percentiles, respectively.

compound induced an increase in the percentage upwind displacement with respect to the control period at all doses tested when applied to the filter papers in dichloromethane ($P<0.05$ in all cases; Fig. 8). In an experiment in which dichloromethane was applied alone to control and test filter papers, the bugs showed no significant change in behaviour ($N=10$). Diluted in paraffin oil, isobutyric acid evoked an increase in the percentage upwind displacement only at the intermediate source dose of 20 µg ($P<0.05$). As with nonanal, paraffin oil also had a retarding effect on the evaporation of isobutyric acid: with dichloromethane as solvent, the concentration of isobutyric acid in the air delivered to the bugs on the servosphere was estimated to be 9 and 245 p.p.b. for source doses of 10 and 100 µg, and in paraffin oil at 5 and 175 p.p.b. for source doses of 20 and 200 µg, respectively. By extrapolation, it can be estimated that the paraffin oil retarded the evaporation of isobutyric acid some threefold. Except for the lowest dose tested (0.2 µg), the upwind response elicited by isobutyric acid (in either dichloromethane or paraffin oil) persisted into the end-control period (comparison of control *versus* end-control periods; $P<0.05$). The target vector, a variable that incorporates both the mean direction of a path and the consistency with which that particular direction is maintained, was also calculated for all treatments. At all doses of isobutyric acid except 20 µg diluted in dichloromethane (which evoked the lowest significant increase in upwind displacement), there was agreement between the increase in target vector and the increase in upwind displacement. This confirmed that quantifying attraction by measuring the displacement of the bugs in the arbitrarily defined upwind cone was satisfactory.

Three different mixtures of isobutyric acid and nonanal were tested using dichloromethane as solvent. One of them, 2 µg of isobutyric acid plus 10 µg of nonanal, induced an upwind response ($P<0.05$, Fig. 8). This was not significantly different from that evoked by 2 µg of isobutyric acid alone either in terms of upwind displacement or in terms of walking speed (two-tailed Mann–Whitney *U*-test), nor did the effect persist into the end-control period. No increase in upwind displacement was induced by this mixture diluted 10 times, by a mixture with each compound at 2 µg in dichloromethane, or by one with 20 µg of isobutyric acid plus 50 µg of nonanal in paraffin oil.

Isobutyric acid diluted in paraffin oil (source dose 20 µg, or approximately 5 p.p.b. in air) combined either with ammonia (source dose 1700 µg of NH_4OH, or approximately 10 p.p.b. in air; Taneja and Guerin, 1997) or with CO_2 (approximately 50 p.p.m. above ambient) elicited no behavioural response ($N=10$ and $N=15$, respectively). Human breath (4%) containing 1500 p.p.m. CO_2 caused an increase in walking

speed over the control (median of the increase 2.1 mm s^{-1} over the control value of 33.9 mm s^{-1}, $P<0.01$, $N=15$), but did not evoke a significant increase in upwind displacement (results not shown).

Comparison of the electrophysiological and behavioural responses to isobutyric acid

By plotting the dose of isobutyric acid in the air delivered to the bugs against the responses obtained in the electrophysiological (data from Fig. 7) and behavioural (data for dichloromethane as solvent from Fig. 8) experiments, a coarse comparison was made between the response dynamics of the acid-sensitive receptor and the behavioural responses to this compound (Fig. 9). To estimate the concentration of isobutyric acid in the air reaching the electrophysiological preparation during the 1 s stimulation, a comparison was made between the response of the acid receptor to a known amount of isobutyric acid eluting from a GC column and the response to the vapour of the acid from different doses in the stimulus cartridge. The amount of vapour leaving the stimulus cartridge is a constant fraction of the dose of product placed there; at a source dose of 1 μg on filter paper, 110 p.p.b. isobutyric acid reached the antennal preparation. Attraction on the servosphere was obtained at between 0.02 and 10 p.p.b. isobutyric acid in the air, i.e. at concentrations that caused low-to-moderate firing (<40 Hz) of the acid-excited receptor in the GP2 sensillum. At approximately 100 p.p.b., a concentration of isobutyric acid that evoked a relatively high spike frequency (>40 Hz), no attraction was observed.

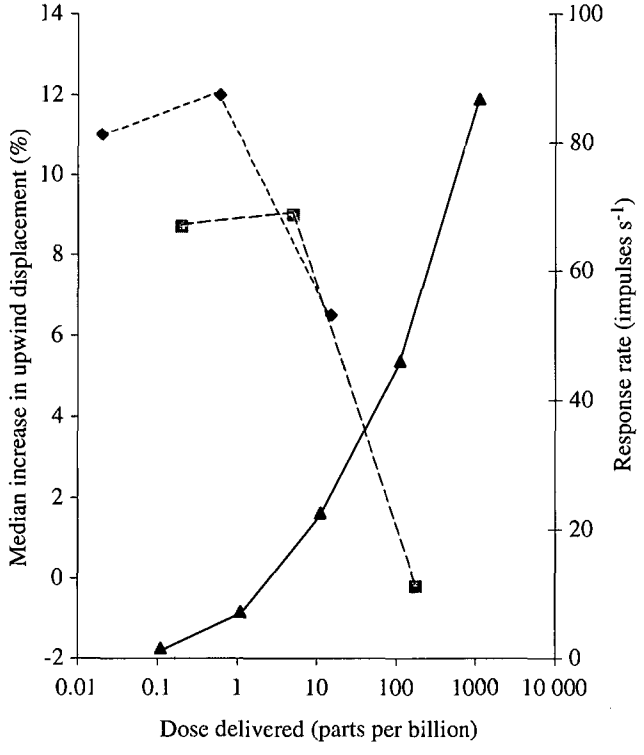

Fig. 9. Comparison of the response dynamics of the acid-sensitive receptor in the GP2 sensillum with the behavioural response to isobutyric acid dissolved in either dichloromethane (diamonds) or paraffin oil (squares) at increasing concentrations (see text for an explanation of estimated amounts of the acid in the air in the electrophysiological and behavioural experiments). Right ordinate, response frequency (triangles; solid line) of the responding receptor cell in the first second of the response after subtracting the blank control value; left ordinate, behavioural response (dashed lines) as medians of the increase in upwind displacement during the test with respect to the control; abscissa, concentrations of isobutyric acid in air delivered to the bugs. The data suggest that attraction occurs within a range of concentrations evoking low-to-moderate responses from the receptor neurone, but not at concentrations eliciting higher firing levels.

Discussion

Electrophysiologically active components of vertebrate odour

Analyses of sheep wool and chicken feather odour extracts using olfactory receptors in basiconic sensilla of fifth-instar *T. infestans* nymphs in GC-EL analyses indicated one biologically active constituent. GC-linked mass spectrometric analyses of these and the rabbit odour extract indicated nonanal as the active constituent of all three electrophysiologically active biological odours. In similar analyses of rabbit odour using receptors in the grooved-peg 2 sensilla, isobutyric acid was shown to be the constituent exciting the acid-sensitive cell in this sensillum type. Both the aldehyde and acid receptors showed selectivity in their responses. The aldehyde receptor of the basiconic sensilla responded to heptanal, octanal and nonanal, but not to longer- or shorter-chain saturated aliphatic aldehydes. Supplementary receptors were activated by nonanal in some basiconic sensilla. This inhomogeneity in the responses between basiconic sensilla is probably related to the high variability in chemosensory innervation of these sensilla in *T. infestans* (M. Vlimant, P. A. Diehl, R. A. Steinbrecht, P. G. Guerenstein and P. M. Guerin, unpublished data; this laboratory). C_7–C_9 saturated aliphatic aldehydes are known to occur in many vertebrate odours other than those reported here, including steer (Steullet and Guerin, 1994a), reindeer (*Rangifer tarandos*) (Müller-Schwarze et al., 1977) and human effluvia (Goetz et al., 1988; Preti et al., 1977). Among haematophagous arthropods, tsetse flies (*Glossina palpalis*) have aldehyde (propanal)-sensitive receptors (Bogner, 1992), and ticks (*Amblyomma variegatum*) possess three types of aliphatic aldehyde receptors (Steullet and Guerin, 1994a). The fact that a receptor responding to nonanal was found in the present study in the basiconic sensilla on the antennae of three triatomine species suggests a role for this compound in the behavioural physiology and chemical ecology of haematophagous reduviids.

It has been established that receptors for NH_3 occur in both GP1 and GP2 olfactory sensilla, and that a short-chain fatty acid receptor also occurs in the GP2 sensilla of *T. infestans* fifth-instar nymphs (Taneja and Guerin, 1997). Here, we have shown that the acid receptor responds best to isobutyric acid (2-methylpropionic acid), followed by the related isovaleric (3-

methylbutyric acid), 2-methylbutyric and butyric acids. The specificity of the response to the C_4–C_5 acids is emphasized by the fact that, although propionic acid occurred in the rabbit odour extract at a level 60 % of that of isobutyric acid, it evoked no response from the acid receptor in any of the GC-EL analyses of this extract. Short-chain fatty acids are commonly present not only in the vertebrate odours tested here but also in the perineal secretions of guinea pigs (Wellington et al., 1979), in the odours of the domestic dog (Preti et al., 1976), in human sweat (e.g. Cork and Park, 1996), in human axillae (Zeng et al., 1991) and in human vaginal secretions (Preti et al., 1977). Lipids are present in a unique mixture of branched and unbranched long-chain fatty acids on the skin of vertebrates, conferring vertebrate-specific skin coatings (Nicolaides, 1974; Korting et al., 1988). Short-chain fatty acids arise from longer-chain fatty acids and also from amino acids *via* bacterial action (Leyden et al., 1981; Albone, 1984; Luckacs et al., 1991). The odours of humans, dogs and guinea pigs, three of the most important hosts of *T. infestans*, all contain a mixture of short-chain fatty acids (Albone, 1984). Isobutyric acid is produced by Brindley's gland in adult triatomines and released from the bugs following mechanical disturbance (Schofield, 1979; Cruz López et al., 1995; see below) and, as such, is referred to as the alarm pheromone of triatomines (Kälin and Barrett, 1975). Among haematophagous arthropods, olfactory receptor cells responding to short-chain fatty acids have been reported for mosquitoes (Lacher, 1967; Davis and Sokolove, 1976; Bowen, 1995; Pappenberger et al., 1996; Meijerink and Van Loon, 1999), for sandflies (Dougherty et al., 1999) and for ticks, in which a receptor specifically tuned to isobutyric acid also occurs (Steullet and Guerin, 1994b).

Behavioural responses to vertebrate volatiles

Nonanal on its own, at a source dose of 10 µg, affected triatomine behaviour on the servosphere. The bugs increased their walking speed, i.e. showed an activation effect, in response to delivery of this compound. The effect ceased once nonanal was removed. Although the amount of nonanal delivered in the electrophysiological apparatus was not quantified, stimulation of tsetse fly antennae with 1-octen-3-ol for 1 s at 1 ml s^{-1} delivers approximately 3 % of the 5 ml stimulus cartridge load for a range of source doses covering those used in the present study (P. G. Guerenstein and C. McMahon, unpublished data). Taking this as an approximation, 15 p.p.b. (representing 3 % of a 0.1 µg source dose) provides a rough estimate of the threshold of the aldehyde receptor. A source dose of 1 µg of nonanal in the behavioural assay, or 1 p.p.b. in air, represented a behaviourally subthreshold dose, whereas a source dose of 10 µg, or 10 p.p.b. in air, a level just under or at the receptor threshold, activated the bugs on the servosphere. That this should be the case for both nonanal and isobutyric acid (see below) is not surprising. Although the increment in the firing rate of any given receptor may be small at threshold levels, the effect across the cohort of such receptors on the antenna sums at the site of convergence of the axons from these chemoreceptors in the insect's brain. Nonanal is known to affect the behaviour of other insects, including haematophagues, serving as a mosquito oviposition stimulant (Du and Millar, 1999), as a sex pheromone in some phytophagous heteropterans (Aldrich et al., 1984; Gough et al., 1986; Aldrich, 1995) and in the wax moth *Galleria mellonella* (Payne and Finn, 1977) and as a component of the aggregation signal of the locust *Schistocerca gregaria* (Torto et al., 1996). Saturated aliphatic aldehydes are constituents of the green leaf odour, and many phytophagous insects, including some heteropterans, use them during host-finding (Visser, 1986; Chinta et al., 1994). From the data obtained in the present study, we suggest that nonanal plays a role as a chemical host cue for triatomines.

Isobutyric acid had a different behavioural effect from nonanal on the triatomines. This acid caused positive odour-mediated anemotaxis by bugs on the servosphere, and the effect generally persisted into the end-control period. These behavioural responses to isobutyric acid were measured at concentrations within and below the electrophysiological response range of the acid-excited receptor. Schofield (Schofield, 1975) found that isobutyric acid attracted triatomines only at 'low' source doses and even repelled them at 'high' doses (>10 mg, see also Ward, 1981). A similar phenomenon was reported for mosquitoes, in which only a moderate increase in the dose of L-(+)-lactic acid over to that evoking maximal attraction caused a dramatic decline in the response (Geier et al., 1999). Parsimony, or the use of the same chemical product as a releaser of different behaviours, is common in arthropods (Blum, 1996). Although isobutyric acid may evoke 'escape' responses when released suddenly at high doses from Brindley's glands of disturbed adults, this product may have another function as a cue to locate a blood meal at host-related doses.

Because nonanal and isobutyric acid were both found to occur in rabbit odour, mixtures of these two compounds were tested. A mixture of approximately 8 p.p.b. nonanal plus approximately 1 p.p.b. isobutyric acid in air induced an upwind response in *T. infestans* nymphs, but the acid content of this blend on its own could account for the effect. The activation effect evoked by the same dose of nonanal when tested alone was not induced by the mixture. Other mixtures of these two products did not induce any behavioural effect, indicating that the dose and ratio of the appropriate mixture of host chemicals are critical for activation and attraction of triatomines.

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TECHNICAL NOTE

A comparison of volatiles emitted by adults of three triatomine species

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Introduction

Adult triatomine bugs (Hemiptera: Reduviidae: Triatominae), blood-sucking insect vectors of Chagas' disease, possess three types of exocrine glands that are absent in nymphs. The paired metasternal glands are located on the ventral metathorax, while the paired Brindley's glands are situated on the dorsolateral metathorax. In addition, glandular areas associated with the male genitalia were recently described (Weirauch, 2003). Nothing is known about the stimulus for metasternal or genitalia-associated gland secretion in triatomines, but disturbance of adult bugs induces the release of an odour, probably from Brindley's glands, that is repugnant to the human nose (Kälin & Barrett, 1975; Schofield, 1975).

It was reported that Brindley's gland secretion and the headspace over disturbed adult *Triatoma infestans* (Klug) consisted of a mixture of carboxylic acids that included isobutyric acid (Hack et al., 1980; Juárez & Brenner, 1981). More recently, Cruz López et al. (1995) analysed Brindley's gland secretion, and the headspace of disturbed adult *T. infestans* and found isobutyric acid, together with a mixture of aliphatic alcohols and esters, and aromatic compounds. No carboxylic acid other than isobutyric acid was detected by them, thus contradicting the findings of Hack et al. (1980) and Juárez & Brenner (1981). Analysis of Brindley's gland secretion of the triatomine *Rhodnius prolixus* (Stål) suggested the presence of isobutyric acid, other carboxylic acids, and three unidentified esters (Rojas et al., 2002).

While the secretion from Brindley's gland was suggested to represent an alarm signal (Kälin & Barrett, 1975; Schofield, 1975; Barrett, 1976; Ward, 1981), the existence of a sex/aggregation signal produced by adult triatomines during copulation has also been reported by different authors (Baldwin et al., 1971 for *R. prolixus*; Manrique & Lazzari, 1996 and Fontan et al., 2002 for *T. infestans*; for a review see Cruz López et al., 2001).

Given the variability of the results obtained for *T. infestans* by previous authors we asked if the blend of odours released by disturbed adult triatomines, supposedly originating in the Brindley's gland secretion, is characteristic of each species. The headspace vapour over disturbed adults of different triatomine species [*R. prolixus*, *T. infestans*, and *Dipetalogaster maxima* (Uhler)] that were reared and tested under similar conditions was analysed here. These tests were aided by the use of solid phase micro-extraction (SPME), a technique that does not require the use of solvents for the analysis of volatiles. In addition, efforts were made to identify any mating-associated odour for *R. prolixus*.

Materials and methods

Colonies of *R. prolixus*, *T. infestans*, and *D. maxima* were maintained in our laboratory as described in Taneja & Guerin (1997). Only adult triatomines were used in the experiments.

Odour collection from disturbed adult triatomines

Groups of eight 3–9-week-old (four males plus four females) *R. prolixus* or *T. infestans* starved for 1 week at 22 ± 1 °C were used. The bugs were introduced into an empty 300 ml Erlenmeyer flask with a Teflon septum and then disturbed by vigorously tapping and shaking the flask for 15 s. The flask was then placed in a climate chamber at 26 ± 1 °C during the bugs' virtual day, and 30 min later a polyacrylate SPME fibre (85 µm film thickness; Supelco, USA) was introduced. Gender differences in the compounds released by disturbed *R. prolixus* were assessed by testing a group of five males and a group of five females as described above. The odour present over a group of three male *D. maxima* (10-week-old

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males, starved for 1 week) was sampled using both the polyacrylate and polydimethylsiloxane (100 µm film thickness) SPME fibres. These animals were disturbed using an agitator at 3 Hz (amplitude of movements: 4 cm) for 15 min at room temperature. No compounds were detected from *R. prolixus* or *T. infestans* using this method.

For all three species, the sampling period by SPME was 50 min, and a control under the same conditions was made just before each test, using the same empty flask. Before use, fibres were conditioned for 2 h at 300 °C in a stream of N_2.

Odour collection from males and mating pairs of *Rhodnius prolixus*

The insects were 3 months old, and had been starved for 3 weeks at 22 ± 1 °C.

Virgin mating pairs. After moulting to the adult stage, 17 virgin females contained in a plastic container (500 ml, 7 cm diameter, 12 cm high), were carefully transferred to another similar clean container using a brush. A folded filter paper disc (125 mm diameter) manipulated with plastic gloves was then introduced into this container, which was sealed with netting. The animals were held in this plastic container for 2 h during which time they spontaneously moved to the filter paper, where they stayed. The same procedure was repeated using 17 males in another container. After the 2-h period, the filter paper disc with females and that with males were carefully introduced into a 1.2 l desiccator (11 cm wide mouth). The initial headspace over the animals was evacuated by passing room air at 250 ml min^{-1} for ca. 6 min before starting the odour collection. Forty min was allowed after closing the desiccator for mating pairs to form before starting the odour sampling. Nine couples copulated during the collection. A control experiment consisted of sampling the air from the glass container with only two filter paper discs just before the test with the triatomine couples.

Non-virgin males. Air from 34 non-virgin males was sampled using the same protocol as for the virgin mating pairs. Five copulation attempts between males were observed during odour collection. Females alone were not tested.

A closed-loop stripping apparatus (CLSA; Grob & Zürcher, 1976) with a 1.5 mg activated charcoal trap held in a 4 mm i.d. glass tube was used to collect the odours. An airflow of 200 ml min^{-1} passed through the desiccator on to the charcoal trap for 30 min. In the CLSA, volatiles breaking through the charcoal trap are restored into the collection loop via the pump. The trap was extracted by passing 16 µl of dichloromethane (DCM, Merck, analytical grade) through the charcoal bed 30 times. After this, 8 µl of extract was recovered, and 2.5 µl analysed by gas chromatography linked mass-spectrometry (GC-MS, see below). Before use, the trap was conditioned by washing it with DCM and heating it at 50 °C in a N_2 stream at 100 ml min^{-1}. These experiments were done at dusk at 23 ± 1 °C.

Gas chromatography coupled mass-spectrometry (GC-MS)

Samples collected on SPME fibres or on charcoal were analysed on a Hewlett Packard 5890 series II chromatograph linked to mass selective detector (MSD; 5971A, ionisation chamber temperature 180 °C; ionisation energy 70 eV, scanning for masses 19–300) using a fused-silica free fatty acid phase capillary column (FFAP, 30 m, 0.25 mm i.d., 0.25 µm film thickness; BGB Analytik, Switzerland). The samples collected on charcoal were injected on-column, and the samples collected on the SPME fibre were injected using the split/splitless injector at 240 °C to desorb volatiles from the fibre for 1 min in splitless mode. The carrier gas was He, maintained under constant flow (velocity 30 m s^{-1} at 40 °C) when injecting on-column, or under constant pressure (50 kPa) when injecting splitless, starting from 40 °C and temperature programmed at 8 °C min^{-1} to 180 °C and 5 °C min^{-1} to 220 °C and held for 10 min. When the headspace over a group of four male and four female disturbed *R. prolixus* or *T. infestans* was sampled, analyses were done using a DBWAX capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, USA). The identification of volatiles was based on the match of the mass spectrum of the natural products with that of a known product stored in the computer-based HP CHEMSTATION library of mass spectra linked to the mass selective detector. The mass spectrum and retention time of a natural product were then compared with those of the library-proposed synthetic equivalent injected under the same conditions.

Results and discussion

The headspace over disturbed *R. prolixus* adults consisted of a blend of five carboxylic acids: acetic, propionic, butyric, 2-methylbutyric, and isobutyric acid (77–91% of the blend; Figure 1a). The identity of the compounds was confirmed by matching the mass spectra and retention times of the synthetic equivalents injected under the same conditions (indicated with an asterisk in Figure 1a). Two of the components of this blend (acetic and isobutyric acid) were also found in the Brindley's gland of *R. prolixus* and were described as triatomine attractants (Rojas et al., 2002). However, we did not find any ester, as reported in the secretion of the gland (Rojas et al., 2002). 2-Methylbutyric acid is reported here from triatomines for the first time. An olfactory receptor cell on the antenna of *T. infestans* nymphs, which is most sensitive to isobutyric acid, also responds to 2-methylbutyric acid (Guerenstein & Guerin, 2001). No

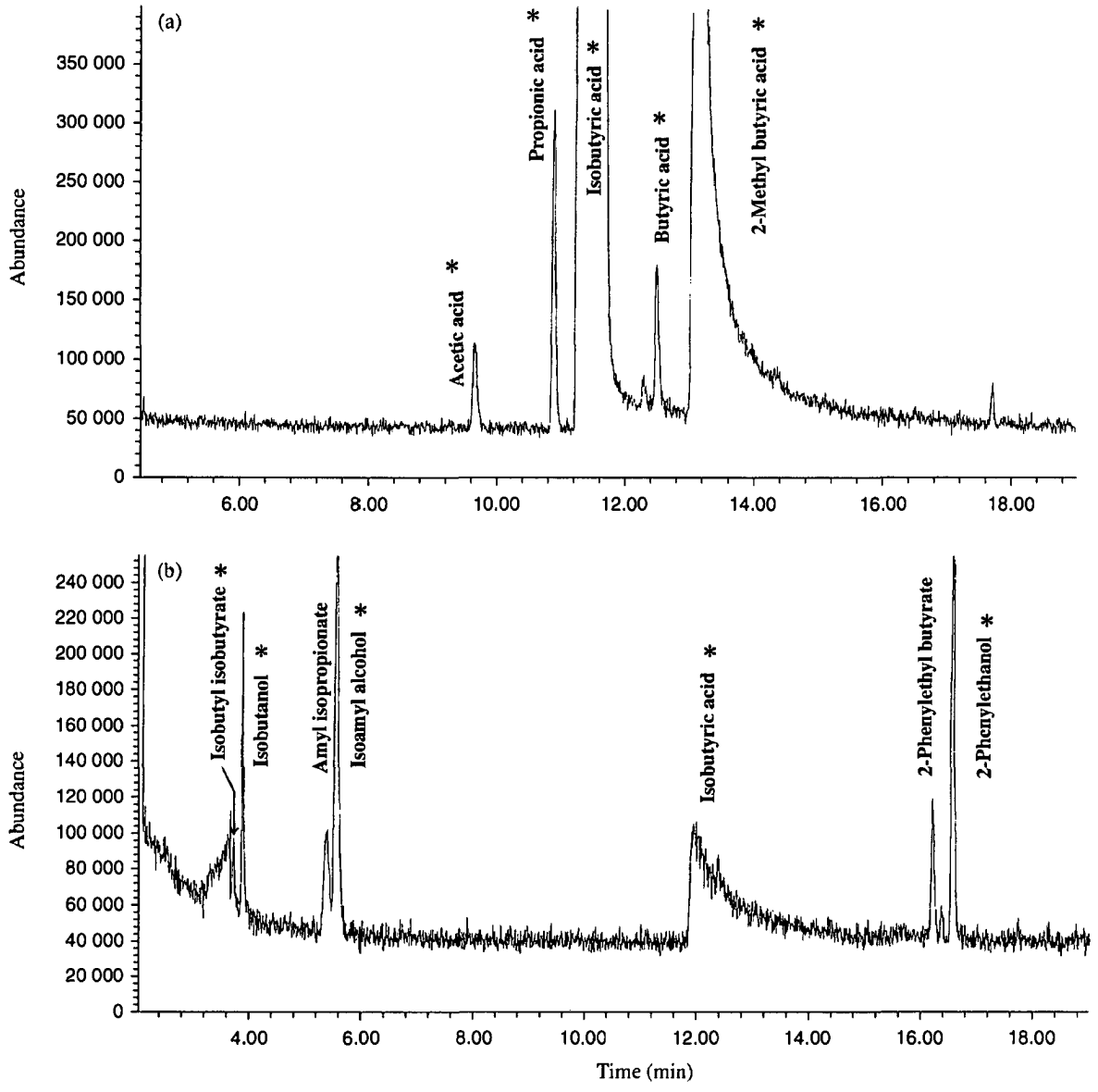

Figure 1 Total ion chromatogram of volatiles collected over eight (four male plus four female) disturbed adult *R. prolixus* (a), and *T. infestans* (b), sampled with a polyacrylate SPME fibre and analysed by GC-MS on a DB-WAX capillary column. Ordinate, total ion count detected by the MSD, and abscissa, retention times of the eluting compounds. Each 1 min interval is equivalent to an 8 °C rise in oven temperature, starting at 40 °C (isobutyric acid elutes at ca. 130 °C). The peaks that were identified represent >3 ng. Compounds whose identity was confirmed by machine mass spectra and retention times of synthetic equivalents are marked with an asterisk. Note that the ordinate scale in (b) is different from that in (a).

qualitative differences between the mixtures released by disturbed male or female *R. prolixus* were found (not shown), in agreement with the findings on Brindley's gland content in *R. prolixus* (Rojas et al., 2002), and as reported for *T. infestans* (Hack et al., 1980; Juárez & Brenner, 1981).

Seven compounds were tentatively identified by GC-MS in the headspace over disturbed *T. infestans* (isobutanol, isoamyl alcohol, amyl isopropionate, isobutyl isobutyrate, 2-phenylethanol, and 2-phenylethyl butyrate, as well as isobutyric acid; Figure 1b). Five of these, for which synthetic equivalents were available (isobutanol, isoamyl alcohol, isobutyric acid, isobutyl isobutyrate, and 2-phenylethanol), matched the retention times of the synthetic products injected under the same conditions (indicated

with an asterisk in Figure 1b). The blend of compounds collected in this study was similar to that reported by Cruz López et al. (1995) but different to that found by Juárez & Brenner (1981). The latter authors detected a mixture of C_2–C_5 acids (acetic, propionic, isobutyric, and isovaleric acid) in the headspace over disturbed adult *T. infestans* – a blend more akin to that found in this study from disturbed *R. prolixus*. Both blends reported from *T. infestans* have a counterpart in the profiles of compounds found in Brindley's gland by different authors (Hack et al., 1980; Cruz López et al., 1995).

Isobutyric acid was the only product common to the headspaces from disturbed *R. prolixus* and *T. infestans* reared and examined under the same conditions. This may indicate that the odour blend released by triatomines after mechanical disturbance is species-specific. However, isobutyric acid is not a universal secretory product among disturbed adult triatomines. GC-MS analysis of the headspace of three disturbed adult male *D. maxima* suggested the presence of 3-methyl-2-hexanone in the samples obtained with both polyacrylate and polydimethylsiloxane SPME fibres. Single-ion analysis using the MSD at the m/z = 73 typical of $[CH_3CHCO_2H]^+$ on these chromatograms detected no isobutyric acid. 3-Methyl-2-hexanone was also found by Rossiter & Staddon (1983) in the metasternal glands of *D. maxima*. It therefore seems likely that, at least in this species, the secretion from this gland functions as a defensive or alarm signal.

The only compound detected in headspace samples over both males alone and mating pairs of undisturbed *R. prolixus* was isobutyric acid. Ríos-Candelaria (1999) also found this compound from mating pairs of *R. prolixus*. In addition, isobutyric acid, together with other compounds, was also found in the headspace from mating *T. infestans* (Fontan et al., 2002). It seems unlikely that the release of this acid in our experiments was due to an inadvertent disturbance of the bugs. Firstly, as mentioned above (see Materials and methods), the disturbance of adult *R. prolixus* had to be relatively strong for detection of compounds in the headspace of the disturbed bugs. Secondly, in an additional series of odour collections (not shown) where the loop of the CLSA was opened, the vibrations generated by the air-pump (the only apparent source of a possible disturbance to the insects during the sampling period) were avoided by placing it in a neighbouring room. In these samples, isobutyric acid was still collected in one out of three experimental groups of 20 insects each, at a level close to the detection threshold of the MSD.

The amount of isobutyric acid from undisturbed males and mating *R. prolixus* pairs was, on average, more than 100-fold smaller than that from disturbed adult *R. prolixus*. This difference was not due to the different odour sampling method. Thus, it can be suggested that the amount of acid per insect released by undisturbed males and mating pairs was considerably lower than that from disturbed insects. This may suggest that the bugs continuously release low quantities of the acid. The release mechanism of the secretion from Brindley's gland has already been described (Barrett et al., 1979). However, it is not known if the gland is hermetic in the 'closed' state, and if not, this could lead to a slow and constant release of the secretion.

Many of the compounds (including isobutyric acid) detected in this study from the headspace of adult triatomines are known constituents of vertebrate odour (Wellington et al., 1979; Albone, 1984; Jemiolo et al., 1994). Isobutyric acid has been considered the 'alarm pheromone' of triatomines (Kälin & Barrett, 1975; Barrett, 1976) due to its prevalence in the headspace vapour over adults of several triatomine species when physically disturbed, and because of its dispersal effect on bugs. That isobutyric acid might induce different behavioural effects as a function of dose was first suggested by Schofield (1975), who demonstrated that high levels of this compound repelled *T. infestans* (alarm signal role) in an olfactometer in still air, whereas lower levels attracted these bugs. Data from experiments on a servosphere suggested that isobutyric acid is attractive at levels that are near the electrophysiological threshold of its olfactory receptor cell (ca. 0.1 p.p.b.), but this attraction disappears at levels 3 log steps higher (Guerenstein & Guerin, 2001).

A few undisturbed adults could release low levels of acid that may cause the animals to aggregate in a refuge, just as similar concentrations of the product may play a role in attracting hungry bugs to vertebrates (Guerenstein & Guerin, 2001). Although the existence of an aggregation pheromone in 'triatomine odour' has not been clarified, it was suggested that during copulation, adult *T. infestans* form aggregations using odour cues for orientation (Manrique & Lazzari, 1996). In addition, undisturbed adult *T. infestans* were reported to attract other adults of the same species even before copulation (Fontan et al., 2002). In ticks, isobutyric acid is the predominant compound of the aggregation-attachment pheromone of *Amblyomma hebraeum* (Koch) (Apps et al., 1988).

A parsimonious use of isobutyric acid (and maybe some of the other compounds secreted by the exocrine glands) by triatomines can be proposed as this compound could be used as an alarm signal, a host attractant, and as an intraspecific aggregation signal, depending on the concentration and physiological state of the perceiving bug. Products that function as alarm pheromones for a species when present in relatively high concentrations, and that promote aggregation when present at lower levels, are known for other insects (Blum, 1996).

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